CHAPTER 9

Stress Proteins: Potential as Multitiered Biomarkers

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INTRODUCTION

The biomarker concept involves the use of biochemical, cellular, and physiological parameters as diagnostic screening tools in environmental monitoring. Although biomarkers have been used in a variety of contexts this article deals specifically with the application of biomarkers for two different purposes: to diagnose sublethal stress in an organism, designated as a tier I biomarker, and to detect exposure to specific contaminants, a tier II biomarker.

Important criteria for evaluating the utility of tier I biomarkers for diagnosing sublethal stress would include: (1) its ability to be used in a broad range of organisms when exposed to a wide variety of stress conditions in their environment, (2) that it correlates with decreased physiological function and survival of the organism, and (3) that, in practicality, it can be easily measured in a cost efficient manner. Tier II biomarkers, used to identify exposure to specific contaminants, should be detected in organisms exposed to a particular class of contaminants in their environment and be easily measured. By integrating both kinds of biomarkers into a multitiered approach to environmental monitoring, one could develop a series of assays in which organisms are initially screened with biomarkers to detect general stress and, if the results were positive, could be assayed with an array of tier II biomarkers, each of which identifies exposure to a particular class of contaminants or physical conditions. Such a strategy

would provide a comprehensive overview of both the extent of biological damage and the "culprits" responsible.

A major advantage of the biomarker approach is that biochemical and cellular events tend to be more sensitive, less variable, more highly conserved and often easier to measure than stress indices commonly examined at the organismic level such as inhibition of growth, changes in rate of development, and reduced reproductive potential. The major disadvantage is that it can be more difficult to relate these biochemical responses to the health of the organism and to adverse effects on the population, the type of information which is often the bottom line in environmental monitoring. We can best overcome this disadvantage if we select as biomarkers cellular and biochemical events which are intimately involved in protecting and defending the cell from environmental insults. It has recently become apparent that one of the earliest cellular responses to environmental stress involves changes in differential gene expression which are part of the cell's attempt to protect itself.[1] In this paper we focus specifically on the potential use of these transcriptional changes as the basis for developing a series of biomarkers for environmental monitoring.

Since biomarkers for general stress should be related to adverse effects on the organism, the mechanisms by which cells actively respond to environmental stress are ideal candidates. However, it is important to keep in mind that one of the most difficult aspects of identifying such primary responses to environmental stress has been the difficulty in distinguishing them from secondary, symptomatic events caused by adverse conditions in the cell as a result of the stressed condition. For example, it is difficult to determine if a shift in energy metabolism is a consequence of the stress event, i.e., the result of the inactivation of key respiratory pigments, or a proactive strategy initiated by the cell to protect itself.

Only very recently have cell biologists begun to understand the molecular mechanisms underlying the physiology of stressed cells.[2] Serious attention to this important cellular phenomena has occurred largely because of the discovery that all cells dramatically alter their gene expression in response to environmental stress. This alteration in transcriptional activity appears to be an attempt to protect the cell from damage and to repair existing damage.[3] This response, initially referred to as the heat shock response because it was discovered upon exposure to a heat shock, is now more commonly referred to as the cellular stress response since it can be elicited by a variety of physical and chemical stressors. Changes in gene expression associated with the stress response are extremely rapid and result in the induced synthesis and accumulation of what is now referred to as stress proteins. Although a few stress proteins appear unique to stressed cells, most are found in much lower concentrations in nonstressed cells where they play a role in normal cellular function.[4] Although the induction of some of these stress proteins is independent of the nature of the stressor, others are quite stressor specific.

Molecular biologists have taken advantage of this heat shock, or stress re-

sponse, as a model system for studying the regulation of gene expression because the response can be easily turned on and off by manipulating environmental factors. Much is known about the multiple levels of regulation of stress protein synthesis.[5] Many of the stress proteins and the genes which code for them have been sequenced in a wide range of organisms and found to be remarkably conserved. Less is known about the biochemical role stress proteins play in stress physiology. However, new biochemical functions are being identified rapidly and there is ample evidence that, in concert, these proteins are involved in protecting the cell from subsequent stress and repairing cellular components.[4,6] Although bacteria, yeast, *Drosophila*, and mammalian cell lines have been used most extensively as model systems, the highly conserved nature of this response allows for broad extrapolation to other organisms. Further, many of the cDNA probes and antibodies developed for these model systems have been found to broadly cross react across phyla.[7]

Stress proteins satisfy many of the conditions of ideal candidates for developing a multitiered biomarker strategy for environmental monitoring.[8] They are part of the primary cellular protective response from environmental stress, are induced by a wide variety of environmental stressors, and are highly conserved in all organisms from bacteria to man.[3] Since a thorough understanding of the cellular function of any potential general stress indicator is essential to relating them to the physiological state of the organism this paper reviews what is currently known about the biochemical and physiological function of the various stress proteins, discusses their role in protection from and adaptation to environmental stress, and evaluates the feasibility of their use as biomarkers for general stress. It also discusses how some stress proteins might be used to determine the chemical and physical nature of the stressor.

THE CELLULAR STRESS RESPONSE

The stress protein response is often broken into two major groups: the "classic" heat shock proteins (hsps) whose synthesis is dramatically increased by heat and a variety of other stressors, and the glucose regulated proteins (grps) whose synthesis is increased in cells deprived of either glucose or oxygen.[4] These two stress protein groups are closely related, having similar biochemical and immunological characteristics and considerable homology exists between families. Interestingly, in many instances the synthesis of these two groups seems to be inversely regulated, i.e., cells deprived of glucose have increased synthesis of grps and a concomitant decrease in the synthesis of hsps.[9] Each stress protein is comprised of a multigene family in which some proteins, termed cognates, are constitutively expressed and others are highly inducible in response to environmental stressors. The stress protein cognates play a role in the cell's basic physiology and are found in unstressed cells.

In this paper we designate a third group of stress proteins, those induced by

a specific set of chemical or physical conditions, the stressor-specific stress proteins. Although these proteins may not be related to one another, they share in common the characteristic that their synthesis is dependent upon the chemical or physical nature of the stressor and is not substantially increased by heat.

The Stress Proteins Induced by Heat: Heat Shock Proteins

This group of stress proteins, the heat shock proteins (hsps), has a high potential as biomarkers for general stress. As it turns out, what had originally been referred to as the "heat shock response", because it was discovered in response to heat, is induced by a variety of other stressors as well. This heat shock response is a fundamental aspect of cellular physiology in which exposure to a stressor results in a dramatic redirection of metabolism such that this suite of stress proteins is rapidly synthesized and the synthesis of other cellular proteins is repressed.[5] The heat shock response has been found in all organisms examined to date and the genes which encode these stress proteins, and the proteins themselves, are remarkably conserved, from bacteria to man.[3]

The hsps are induced by a wide variety of stressors including heavy metals,[10,11] xenobiotics,[12] oxidative stress,[13] anoxia,[14] salinity stress,[15] teratogens,[16,17] and hepatocarcinogens.[18]

The Classes of HSPs and Their Cellular Function

Although the number of hsps induced by heat shock and their exact size are both tissue and species specific, five "universal" stress proteins are found in all eucaryotes. Four of these are referred to by their apparent molecular weight on SDS-polyacrylamide gels: hsp90, hsp70, hsp58, and the low molecular weight hsp20-30. In eucaryotes each hsp is comprised of a multigene family, the members of which are regulated by different promoters and code for closely related protein isoforms.[5,19,20] The fifth hsp is an 8 kDa protein, called ubiquitin. Most of these proteins are synthesized at high levels in stressed cells. However, with the exception of the 72 kDa protein, a highly inducible member of the hsp70 family, all of these proteins are also present in much lower concentrations in unstressed cells.[4] The initial observations that many hsps are found in "normal" cells and that hsp20-30 are developmentally induced in larval systems lead to the suggestion early on that hsps play a role in normal cellular activities. In this section we will discuss what is currently known about the function of each of these protein families in the nonstressed cell, the roles they may play in the physiology of cells experiencing stress, and their potential use as biomarkers.

hsp90. The hsp90 protein is also referred to as hsp83 or hsp89 depending upon the species under study. In mammalian cell culture hsp90 has been found to associate with a number of cellular proteins, including steroid receptors,[21,22] several kinsases,[23] and a number of retrovirus encoded oncogene proteins many of which are tyrosine specific protein kinases.[4,24] The unifying functional theme

in each of these cases appears to be that the binding of hsp90 to these cellular components regulates their activity by preventing them from carrying out their normal functions. In mammals hsp90 is abundant in cells under normal conditions and its synthesis increases about three- to fivefold upon exposure to stress.[4] In light of its normal function, this protein may participate in redirecting cellular metabolism in stressed cells through such mechanisms as the alteration of signal transduction. Given the normal abundance of hsp90 and its limited induced synthesis upon exposure to stress, it may not, by itself, have a great deal of potential as a biomarker.

hsp70. There are two major members of this most highly conserved stress protein, the hsp70 family, each of which is present in multiple isoforms.[5] The larger protein of the two, 73 kDa in mammals, is often referred to as the hsp cognate because it is found in unstressed cells and also exhibits a marked increase in synthesis upon exposure to various stressors. The smaller protein, 72 kDa, is only synthesized upon exposure to stress and is not found in the cell under normal conditions. Although these two hsps are closely related and have similar biochemical properties, they are distinct gene products.

As for the functional aspects of these important proteins, a general picture is now emerging in which hsp70 acts to either stabilize or solubilize a target protein.[4] Under normal conditions such binding may serve a "chaperone" function for newly synthesized secretory and organellular proteins by helping them to translocate across a membrane.[25,26] Another member of the hsp70 family, called BiP or grp78 (discussed below), is also found under normal conditions and has recently been shown to be transported into the endoplasmic reticulum where it may perform a similar chaperone function for proteins transported into this compartment.[27]

The highly inducible hsp72 in conjunction with the other hsp70 proteins may perform a similar role in cells experiencing stress. A major feature of stressed cells is the loss of integrity of the nucleolus and the associated inhibition of rRNA synthesis and ribosomal assembly.[4] Under stress hsp72 rapidly migrates to the nucleolus where it is speculated to resolubilize denatured pre-ribosomal complexes and help restore nucleolar function during recovery from stress.[28] During recovery it migrates to the cytoplasm and associates with ribosomes and polyribosomes where it is speculated that it may bind to denatured proteins and in an ATP dependent manner, facilitate their resolubilization.[28,29] Since the hsp70 family accounts for much of the translational activity in stressed cells and it is one of the most highly conserved proteins known in biology, it is an excellent candidate for a biomarker for general stress.

hsp60. The hsp60 protein, found in the mitochondria, is believed to be another "chaperoning" protein which facilitates the translocation and assembly of oligomeric proteins into that compartment.[30] It is homologous to the bacterial hsp GroEL and the Rubisco-binding protein of chloroplasts,[31] forms large ag-

gregates in the matrix of the mitochondria, and is essential for the assembly of oligomeric complexes imported into the mitochondria. Its synthesis is increased in stressed cells where it is believed to perform similar functions. Because it is highly conserved and its synthesis is increased in stressed cells, it is also a good candidate as a biomarker.

hsp20-30. The low molecular weight stress proteins (hsp20-30) are the least conserved of the stress proteins. They show homology to the alpha crystalline lens protein, and also share with that protein the tendency to form higher ordered structures.[32] These stress proteins are highly species specific. One protein of this class is found in yeast (26 kDa) and mammals (28 kDa), *Drosophila* has four (28, 26, 23, 22 kDa), and some plants have been found to have up to 20 proteins in this class.[5] Considerable variation exists even within the same class of organisms. For example, within the molluscs *Mytilus edulis* has two low molecular weight stress proteins (29, 32 kDa; Figure 1), *Collisella scabra* has at least five (16, 17.5, 18.5, 19, 24 kDa; Figure 2) and *Collisella pelta* has two (17.5, 18.5 kDa).[33] Although low molecular weight hsps are regulated during development and differentiation, and by the hormones estrogen, progesterone, and ecdysone, we know very little about the function of these proteins in cells under normal conditions.[4,5,34,35] However, they do localize within the nucleus upon heat shock and return to the perinuclear region of the cytoplasm upon recovery.[36] Since they are highly species specific and regulated by a number of factors besides exposure to stressors, their use as a biomarker of general stress may be misleading and of limited value.

Ubiquitin. Ubiquitin is a small molecular weight (7 KDa) protein found in all eucaryotic cells. Under normal conditions it is involved in the nonlysosomal degradation of intracellular proteins.[20] When ubiquitin is conjugated to proteins by a ubiquitin-protein ligase system these proteins are selectively degraded. Ubiquitin synthesis increases with exposure to heat and is an essential component of the stress response.[37] In stressed cells the function of this protein would complement the resolubilization and stabilization function of hsp70 by targeting denatured proteins for degradation and removal. In yeast the polyubiquitin gene which is transcribed in stressed cells has been shown to be essential for resistance to heat shock, starvation, and other forms of physiological stress.[37] Because of the inability to detect proteins of such low molecular weight using standard electrophoretic gels, little data are available that would aid in evaluating this stress protein as a biomarker. However, given that the generation of denatured proteins is a common problem in stressed cells, increased synthesis of this stress protein is probably ubiquitous in eucaryotic systems making it a good candidate as a biomarker for general stress. Further, since such damage is quite localized it is unlikely that its synthesis will be regulated by extracellular signals.

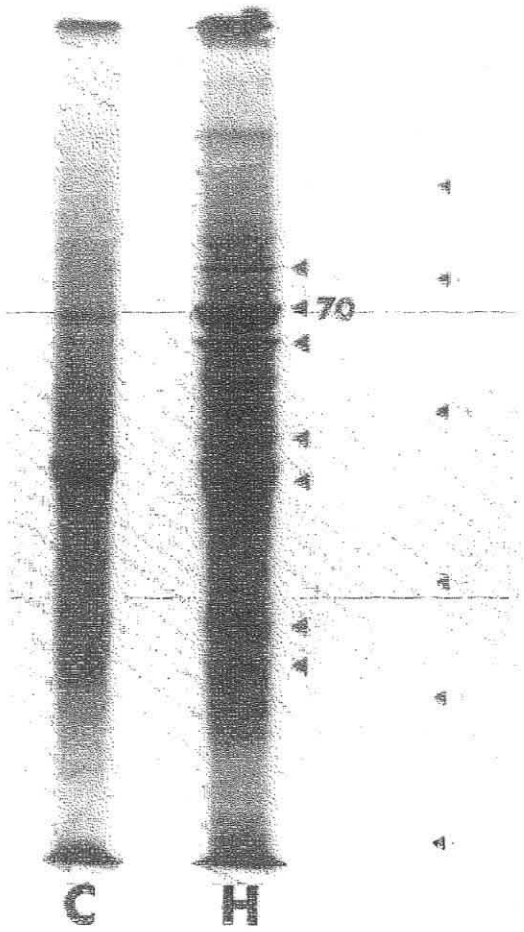

Figure 1. The stress protein response in gill tissue of *Mytilus edulis*.[8] The control (C) was maintained at 17°C, while the heat shock (H) sample was exposed in vivo to a heat shock for 1 hr at 31°C. Tissues were then incubated in ^{35}S methionine, homogenized and equal protein was loaded for one-dimensional electrophoresis (SDS-PAGE). The gels were subsequently fluorographed. Arrows at the far right indicate molecular weight markers of 130, 75, 50, 39, 27, and 17 kDa, respectively. Arrows next to the heat shocked sample indicate stress proteins of 80, 74, 72, 60, 47, 43, 32, and 29 kDa, respectively.

The Glucose-Regulated Proteins

The major glucose regulated proteins (grps) are of 100, 80, and 75 kDa and are structurally and functionally related to the heat shock group. The 75 and 80 kDa proteins are homologous to the hsp70 family,[38] and the 100 kDa protein is homologous to hsp90.[40] This group of stress proteins is present in unstressed cells and shows increased synthesis in cell cultures deprived of glucose or oxygen, exposed to elevated lead,[40] and agents that perturb calcium homeostasis.[4] Since

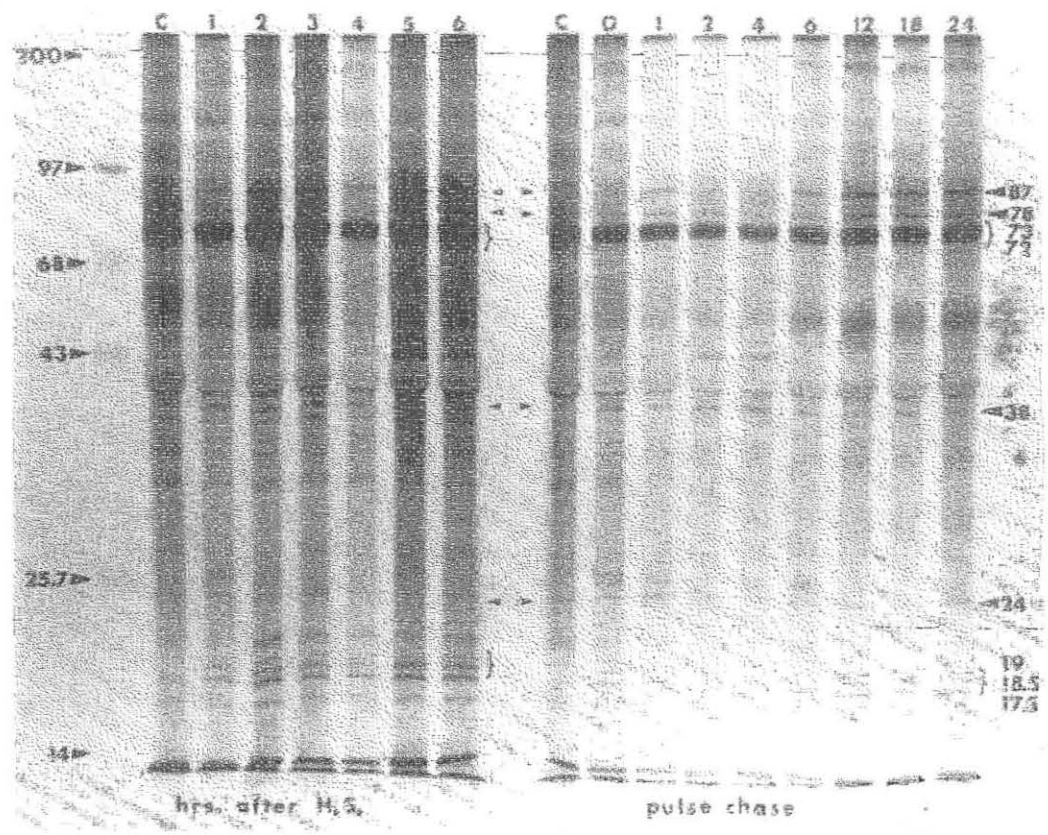

Figure 2. Fluorograph of *Collisella scabra* mantle tissue from induction and half-life experiments. Samples were processed as described in Figure 1. Arrows at the right of the fluorograph identify hsps of 87, 78, 73, 38, 24, 19, 18.5, and 17.5 kDa, respectively. Molecular weight markers are shown on the left. *Left:* Incorporation of ^{35}S methionine into proteins in *C. scabra* mantle tissue at different points in time after a 1-hr, 31°C heat shock. Limpets were heat shocked in vivo and incubated for 1 hr at the times indicated to examine how long the response was induced after heat shock. The control (C) was not heat shocked. Increased synthesis of stress proteins can be seen for at least 6 hr after heat shock. *Right:* Incorporation of ^{35}S methionine into proteins in *C. scabra* mantle tissue during a pulse chase experiment to examine the half-life of the stress proteins. Limpets were heat shocked for 31°C for 1 hr, immediately pulsed with ^{35}S methionine for 1 hr, and incubated in cold methionine over time. The numbers above the sample represent the hours that the tissue was incubated in cold methionine after heat shock. The control (C) was not heat shocked. Stress proteins remain prominent 24 hr after heat shock.

very little is known about the induction of synthesis of these stress proteins in whole organisms, their potential as biomarkers is unclear. Given the specific conditions of increased synthesis of the grps, they appear to be poor candidates as biomarkers for general stress. However, they may have potential as biomarkers to identify anoxic conditions. Also, if the synthesis of grps is increased in starved organisms they may prove useful as biomarkers for evaluating nutritional state.

100 kDa. This grp is found in the endoplasmic reticulum and golgi.[9] That it is homologous to hsp90 suggests it too may be involved in regulating the activity of various cellular components within these cellular compartments under normal conditions. Its amino acid sequence indicates that it is a transmembrane bound protein with much of the protein found on the cytosolic side of the membrane.[41]

80 kDa. This protein, as mentioned earlier, is also referred to as BiP or grp78. It is abundant in normal cells within the endoplasmic reticulum. Its homology with hsp70 suggests that it is involved in the ATP-dependent stabilization, folding, and assembly of proteins transferred into this compartment.[42]

75 kDa. Grp75 is a recently discovered stress protein which is located in the mitochondria. It is another ATP binding protein homologous to hsp70 which probably serves the same protein stabilization and assembly function as the other members of this family.[43]

The Protective Role of Stress Proteins

Collectively these two groups of stress proteins appear to be involved in the protection, enhanced survival, and restoration of normal cellular activities in stressed cells.[44] The induction of hsps by a mild conditioning stress enhances the tolerance of the cell to subsequent, more severe stress situations, a phenomenon often referred to as thermotolerance or, when other stressors are involved, "acquired tolerance".[45–50] The induction, expression, and decay of acquired tolerance correlates with the induction, accumulation, and degradation of stress proteins.[46,51–55] The fact that other studies have demonstrated thermotolerance in the absence of stress protein synthesis,[56–58] has resulted in considerable confusion regarding the role of hsps in thermotolerance. However, Welch and Mizzen[6] have shown that most of these stress proteins are abundant in unstressed cells and rapidly relocate to other subcellular compartments upon heat shock. This observation suggests that even in the absence of protein synthesis these constitutive hsps can be called upon to perform new protective and repair roles under such situations.[4] Although the molecular bases for acquired tolerance is not known, both RNA processing[59] and translation activity[60] appear to be protected by stress proteins. Also lesions caused by heat-shock treatment are prevented or repaired more quickly in cells that have been made thermotolerant by hsp synthesis.[6]

Both the hsp70 class and the low molecular weight stress proteins have been implicated in the enhanced survival of cells exposed to stress.[45,61,62] Hsp70 plays a role in the recovery of nucleoli after heat damage[28,63] and may be involved in the resolubilization of proteins damaged by heat.[29] The accumulation of hsp70 is also correlated with a decrease in the inhibition of protein synthesis which occurs upon severe heat-shock.[60] Mammalian cell lines selected for survival at high temperatures constitutively synthesize hsp70 at high levels while temper-

ature sensitive mutants are unable to elicit the stress protein response.[5] The hsp20-30 family correlates with acquired tolerance in *Drosophila*[47] and sorghum,[64] and accumulates in desert succulents acclimatized to high temperatures.[65] Also, a temperature sensitive mutant of *Dictoselium* is unable to synthesize low molecular weight stress proteins.[61]

In a recent study we examined the heat shock response in two closely related limpet species, *Collisella scabra* and *Collisella pelta,* which have very different tolerances to elevated temperatures that correspond to different microhabitats in the intertidal.[33] We found both quantitative and qualitative differences in the heat shock response. The temperature range for induction of the response reflected the temperature mortality curve for each species, i.e., temperature tolerant *C. scabra* elicited the response and synthesized hsps at higher temperatures than temperature sensitive *C. pelta*. Further *C. scabra* synthesized the highly inducible hsp70 at a higher rate and had many more low molecular weight hsps than *C. pelta*. Boon-Niermeijer et al.[66] have demonstrated that synthesis of stress proteins correlates with acquired tolerance (as assayed by survival) in the mollusc *Lymnaea stagnalis* lending further support to the premise that stress proteins may be involved in enabling these marine invertebrates to survive in the environmental extremes of the intertidal zone.

Stressor Specific Stress Proteins

This third class of stress proteins has in common the fact that its synthesis is induced only under specific chemical or physical conditions. These proteins appear to participate in specific biochemical pathways involved in the metabolism of chemicals, metabolites, or harmful by-products that are the result of a particular chemical or physical condition rather than being part of the cell's protective system in response to general cellular damage. As a consequence, this class does not encompass a homologous group of proteins and is not related functionally or structurally to either of the other two groups of stress proteins. Although numerous inducible enzyme systems (i.e., cytochromes) could technically fall within such a broad definition, this paper will focus on proteins whose kinetics of induction and recovery are rapid (minutes to hours) and are thus part of the cell's immediate stress response. Novel stressor-specific stress proteins are being discovered at a rapid rate; however, most of these are currently only identified by their apparent molecular weight on an SDS gel. We know little about their structure or function. Therefore, this section will be limited to those stressor specific stress proteins which are best characterized, heme oxygenase and metallothionein.

Heme oxygenase

In the last few years a 32 kDa stress protein that was inducible by metals, sodium aresenite, and thiol-reactive agents had been reported in the literature.[11,40] This protein has recently been isolated and identified as heme oxygenase, an

enzyme essential for heme catabolism that cleaves heme to form biliverdin, which is subsequently reduced to bilirubin.[67,68] Although it is considered by some to be a minor hsp because of limited evidence that it can be induced to some extent by heat in rats and humans,[68–70] others report that heat treatment has no effect on heme oxygenase activity or mRNA levels.[71,72] It is most highly inducible by a variety of stressors which cause oxidative damage, such as UVA radiation, sodium arsenite, and hydrogen peroxide.[68] It has been suggested that since breakdown products of heme can readily react with peroxyl radicals they may play an important role in protecting cells from oxidative damage as free radical scavengers in concert with glutathione.[68] Interestingly, Cd and other metals such as Cu, Zn, Pb, sodium arsenite, and gold have been shown to be particularly effective inducers of heme oxygenase and it is the most prominent stress protein induced by these metals upon in vitro exposure.[11,40,68]

Metallothionein

Metallothionein is a low molecular weight ($\geq$10 kDa) cysteine rich metal binding protein whose synthesis is induced upon trace metal exposure.[73] The kinetics of induction tend to be slower than that of the other "classic" stress proteins. For example in trout, metallothionein synthesis is increased within 24 hr of exposure to transition metals while stress protein synthesis increases within 30 min.[74] Although its synthesis is not coordinately induced with the other stress proteins I have chosen to include a discussion of metallothionein in this section because of its role in protection from metal toxicity[73–75] and because its potential in environmental monitoring has been studied extensively.[76–78]

This metal-binding ligand appears to be part of a cellular compartmentalization/sequestration system which evolved to regulate the uptake and tissue distribution of essential trace metals such as the transition elements Zn and Cu.[79] Since the nonessential metal Cd is also a transition element with a similar chemistry to Zn and Cu, it also interacts with this system.[80] Under normal conditions this compartmentalization system provides the cell with ready access to essential metals. However, transition metals are highly toxic and capable of damaging cell function by nonspecific binding to metalloenzymes and other cellular components. When cells are exposed to elevated metals this system also protects the cell by either sequestering the metals in membrane-bound vesicles or on soluble metal binding ligands such as metallothionein and glutathione.[81]

Initial studies suggested that the induction of metallothionein might be a successful biomarker for metal exposure and perhaps even for toxic effects in cases when metals were the major stressor.[78] However, for several reasons metallothionein should be used cautiously as a monitoring tool and is, perhaps, most useful when used in association with other biomarkers. First, it is now clear that, at least in mammals, insects, and crustaceans, metallothionein is induced under many other conditions besides metal exposure, including extracellular signals such as steroid and peptide hormones, interleukin II, and inter-

feron.[82] These and other observations suggest that in many organisms metallothionein may be used by the cell in a proactive manner to regulate metal compartmentalization and accomodate metabolic needs. As a consequence, in higher organisms the increased synthesis or accumulation of metallothionein does not necessarily reflect a reaction to increased metal exposure. Secondly, since metallothionein is only one component of the compartmentalization/sequestration system which regulates metal metabolism, there is no mechanistic basis to assume that measuring metallothionein accumulation without examining the other essential components that regulate metal metabolism (and the metals themselves) will provide reliable information on either metal exposure or the physiological condition of the organism. However, an examination of metal uptake and subcellular distribution has proven quite useful in evaluating organismal stress in the laboratory and in situ.[83–86.]

CONSIDERATIONS FOR EVALUATING STRESS PROTEINS AS BIOMARKERS

Organisms can be exposed simultaneously to many stressors, both natural and anthropogenic, and have only a finite capacity to adapt to them. Yet in environmental monitoring we often look only at one potential stressor out of context with the multiple stressors with which the organism must cope in its environment. Tier I stress responses, those that occur regardless of the nature of the stressor, can be used to evaluate this integrated stress load and determine its overall impact on the physiological state of the organism. However, due to the lack of specificity general responses make it difficult to determine what factors are responsible for the observed stress to the organism. The second tier of stress responses are those which are stressor specific in that they only respond to a specific stressor, or group of stressors, which share chemical or physical characteristics. Although these later responses are less integrative, they facilitate the establishment of cause effect relationships between biological impact and exposure regimes in the environment.

A monitoring strategy using biomarkers would be most effective if it incorporated tier I, general stress responses for initial screening, with tier II, specific stress responses to help identify the stressors. As discussed in previous sections stress proteins have the potential for providing both types of information. Accumulation of the highly inducible hsp72 and hsp60 may provide a useful tier I biomarker which would reflect the integrated stress load on the organism regardless of the type or number of stressors that might be involved. When the accumulation of one of these stress proteins is calibrated to organismal indices of stress, such as inhibition of growth or reproduction, it could be highly predictive of adverse impacts on the population. Once assays based on antibodies raised against these stress proteins are developed to measure stress protein con-

centration, organisms could be screened rapidly to determine if, and to what degree, they are stressed. If stress proteins have not accumulated, no further testing would be necessary. Accumulation of one or more of the grps may also be used as a supplemental assay to screen for nutritional imbalances which might exacerbate the adverse effects of environmental stress on organismal physiology.

The hsps, grps, and stressor-specific stress proteins all have the potential to play a role in the tier II assays which would examine the nature of the stressors involved. By identifying unique patterns of accumulation of the various stress proteins induced by key classes of contaminants we could develop a series of antibody based assays that would allow us to screen for exposure to each type of stressor. As with the general stress proteins, laboratory calibrations between accumulation of particular proteins and organismal stress would provide insight into the relative contributions of each stressor to the entire stress load.

Since the criteria for evaluating biomarkers will be different for each purpose some stress proteins will be more suitable for one application than another. In this section we will examine what is currently known about the use of stress proteins as biomarkers and discuss the kinds of experiments that are needed in order for us to accurately evaluate their potential in environmental monitoring.

Tier I: Stress Proteins as Biomarkers for General Stress

Environmental Relevance

In order to evaluate the potential of using stress proteins as the basis for developing a screening strategy in biomonitoring several characteristics of the response need to be determined. Since an ideal tier I biomarker would evaluate the impact of environmental stress on native organisms and populations in situ, elevated stress proteins must accumulate in organisms under realistic environmental conditions in response to a variety of stressors. Thus, the response should be sustained over time in a variety of organisms upon exposure to sublethal concentrations of stressors.

Unfortunately, to date we know little about the environmental relevance of the stress response and much research needs to be focused in this area. With the exception of metallothionein, much of the research on the stress response has involved exposure of cells in culture to perturbations which are often extreme and unlikely to occur in the environment.[6,62,87] Metabolic labeling studies in our laboratory have demonstrated induction of the stress response at concentrations as low as 7.5 ppt (ng/L) for tributyl tin (TBT) and 10^{-12} *M* free cupric ion activity following a 4-hr in vitro exposure of *M. edulis* hemolymph (Figure 3). These concentrations are well within the ranges measured in the environment. Tributyltin, the active component of some antifouling paints, has been reported to range from <0.04–0.35 ppb near the entrance to a harbor in England.[88] It has been shown that molluscs are particularly sensitive to TBT; concentrations as low as 1 ppt results in 'imposex', the induction of male sexual characteristics

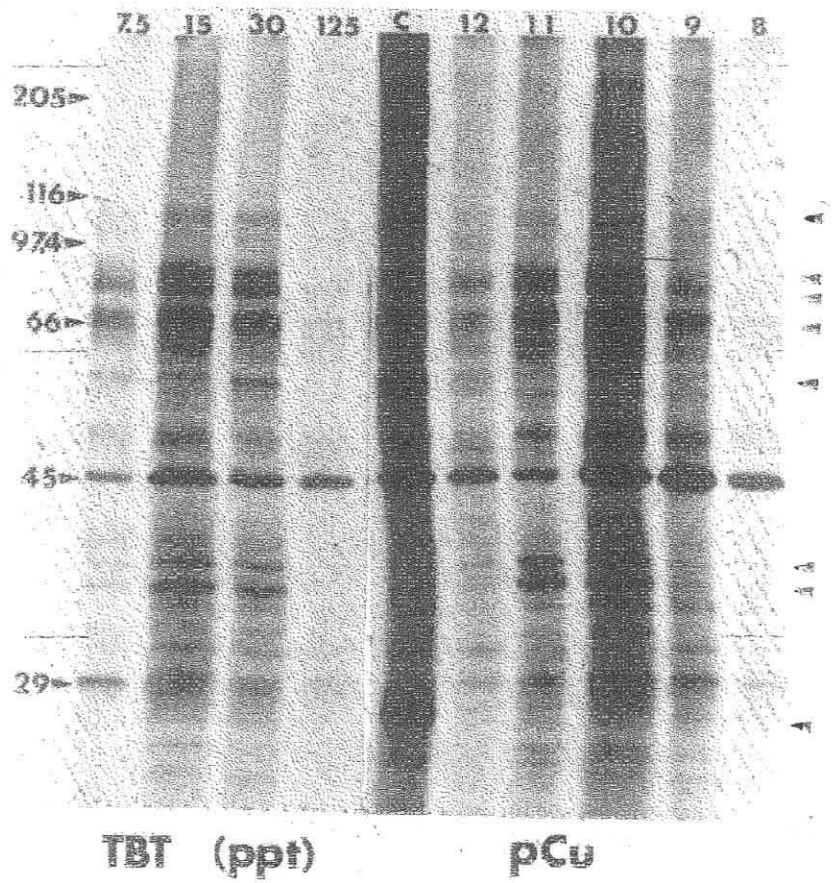

Figure 3. Induction of the stress response in hemolymph of *M. edulis* exposed to tributyltin and copper. Samples were exposed in vitro to the concentrations indicated for 4 hr and then incubated for 4 hr in ^{35}S methionine. Samples were processed as described in Figure 1. Arrows at the left are for the molecular weight markers. Those on the right indicate stress proteins of approximately 110, 85, 78, 74, 72, 60, 38, 36, and 27 kDa, respectively. TBT = tributyltin, ppt = parts per trillion, pCu = $-\log$ of free cupric ion concentration.

in females[89] and the 15-day larval LC_{50} for tributyltin oxide is 0.1 ppb.[90] Free cupric ion activities have been measured as high as $10^{-10.5}$ *M* in several estuaries in North Carolina, a concentration at which growth is inhibited in crab larvae.[53]

Further, recent studies have indicated that the stress response is induced in vivo at concentrations found in polluted environments. Irby et al.[12] have demonsrated several-fold increases in accumulation of hsp60 in rotifers after 96-hr exposures to concentrations as low as 15 ppb (μg/L) of TBT and 10 ppb (μg/L) Cu. In experiments recently completed in collaboration with scientists at the EPA/ERL laboratory in Narragansett, Rhode Island, we found significant increases in hsp60 concentrations in *M. edulis* mantle tissue after a 7-day in vivo exposure to Cu low as 3.2 ppb (μg/L).[33] Thus, it does appear promising that the stress response is elicited under environmentally relevant concentrations across a broad range of organisms.

If it is to be useful for native organisms exposed in situ the stress response must also persist over time. Although reports in the literature are contradictory most researchers now estimate that hsp70 has a half-life of at least 4 days in most organisms.[5] Pulse chase experiments in this laboratory have shown stress proteins to be stable for at least 72 hr in marine molluscs (Figure 2).[33]

Data on the kinetics of induction and recovery of the stress response suggest differences which are specific to the type of environmental insult and severity of the stress. In mammalian cells a 42°C heat shock induces the response within a few minutes and recovery, as determined by a reversion back to translational patterns similar to controls, occurs 4 hr after removal of the heat shock.[70] Although the kinetics of induction are similar in response to sodium arsenite, recovery is not apparent until 8 days after removal of the stressor, presumably because the stressor is still present in the cell. Surprisingly, marine invertebrates which are notorious for their slow metabolism, display similar induction and recovery kinetics. In the mollusc *C. scabra* hsps are rapidly synthesized within minutes of a 31°C heat shock and the organism continues to synthesize stress proteins for at least 6 hr after removal from elevated temperatures (Figure 2).

In a broad range of organisms continuous exposure to a contaminant which results in moderate stress appears to elicit a transient response, while continuous exposure to a contaminant which results in a more severe stress results in a sustained response.[5] One concern in terms of the usefulness of the stress response in biomonitoring is that if chronic environmental exposure resulted in a less severe stress, and thereby a transient expression of the stress response, the organism may be experiencing stress yet stress proteins may not be significantly elevated over time. If this was the case it would limit their usefulness as biomarkers for in situ exposure of native organisms.

It is important, however, to distinguish between the stress response, which is defined as the increased rate of synthesis of stress proteins and inhibition of the rate of synthesis of normal proteins as detected through translational patterns, and significant increases in accumulation of stress proteins which are persistent in time. Mizzen and Welch[60] have demonstrated that hsp70 expression is regulated in such a way that regardless of the number and severity of heat shock treatments its synthesis is increased until a threshold concentration of the protein accumulates in the cell. After this point no amount of stress will result in an increase in hsp70 concentrations. Given what we now know about the function of this protein it is not surprising that it is the total pool of this protein which is being regulated. As a result of this regulation it is also quite likely that under chronic stress conditions elevated stress proteins persist in time by balancing the rate of synthesis against the rate of degradation. If this is the case elevated stress proteins would persist even though the translational patterns characteristic of the stress response would not be observed.

Although numerous laboratory and field experiments are needed before we will be able to evaluate this aspect of the response, Kee and Noble[65] have demonstrated that low molecular weight stress proteins remain abundant in desert

succulents even after completion of high temperature acclimation. In *M. edulis* we have observed sustained elevated accumulation of hsp60 for at least 1 week upon exposure to Cd and to Cu.[33] Clearly long term experiments need to be carried out to determine if elevated accumulation of stress proteins is a chronic condition upon exposure to sublethal stress.

Relationships to Physiological State of the Organism

A major advantage of the stress response is that because it is involved in protecting the cell from environmental damage, it provides a direct measure of the cellular physiological state. Thus it has the potential to be more sensitive than existing organismal indices for stress, yet can be correlated to adverse physiological conditions in the organism. Further, as a quantitative response, it could provide the added benefit of evaluating the extent to which an organism is stressed. To evaluate this aspect of the stress response more research is needed that examines the relationships between stressful conditions at a tissue level and the physiological condition of the organism.

A few studies have attempted to begin to examine these relationships. In collaboration with EPA scientists we have attempted to correlate Cu exposure in *M. edulis* to tissue specific increases in hsp60 concentration and to scope for growth (SFG), a common organismal stress index based on bioenergetic changes which measures clearance rate, respiration rate, and assimilation efficiency.[33] In these experiments, groups of organisms were exposed to Cu concentrations of 0, 3.2, 10, 32, and 100 ppb for 7 days. One half of each treatment ($n = 8$) was then used for scope for growth measurements and the other half for hsp60 accumulation. The SFG index showed no significant differences among organisms exposed to 0, 1, 3.2, and 10 ppb Cu. However, for those exposed to 32 and 100 ppb the SFG was significantly lower than that for controls, a condition indicative of growth inhibition. Dramatic reductions in clearing rates across the gills seemed to be a major factor in this reduction. When we measured the accumulation of hsp60 in mantle tissue we found no difference in its accumulation between controls and 1 ppb Cu. However, hsp60 concentrations were significantly higher at all other Cu concentrations. There was a linear relationship between the log of the Cu concentration and the log of hsp60 concentration in tissues exposed to 3.2 to 100 ppb Cu. Further, we could detect stress at the tissue level, defined by a significant increase in hsp60, at a Cu concentration one order of magnitude lower than could be detected with scope for growth.

Assays to Measure the Stress Response

On a practical level an ideal biomarker should be easy to use, rapid and inexpensive and would measure a response which is so highly conserved that a single probe could be used with a broad range of organisms, vertebrates, invertebrates, and plants alike, so that native organisms could be examined in a site specific manner.

The techniques most frequently used in the study of stress proteins have

involved metabolic labeling and specific antibodies or cDNA probes. In metabolic labeling studies tissues are incubated with an amino acid tagged with a radioisotope (i.e., ^{35}S, ^{14}C, ^{3}H). The tissue is then homogenized and the proteins are separated by one- or two-dimensional electrophoresis, and autoradiographed to examine incorporation of the radioisotope into specific proteins (Figures 1, 2, 3). This technique provides information on the entire translational profile in response to a stressor and can be particularly useful for identifying new inducible proteins. However, under continuous exposure to moderate stress conditions these dramatic changes in translational patterns are transient (approximately 18 hr in *Mytilus* exposed to a mild heat shock) and translational activity reverts to patterns similar to those found in controls. Therefore, it appears likely that it will be the stress protein concentration which will be most reflective of stress in organisms exposed to moderate perturbations over long periods of time. Since there is currently no simple quantitative assay for measuring any of the stress proteins these techniques will need to be developed before extensive research on the quantitative aspects of the stress response can be carried out.

Assays based on antibodies raised against the stress proteins have the best potential for environmental monitoring because they will allow us to directly quantify stress protein accumulation. Of all the stress proteins the hsp70 family is an ideal candidate for an assay of general stress because it is highly inducible and is found in high concentrations in tissues of organisms exposed to environmental perturbations. Further, since it is so highly conserved there is the potential of developing antibodies which may crossreact in a broad range of organisms. A number of antibodies have been raised against the two hsp70 gene products isolated in mammals, *Drosophila*, yeast, and bacteria. However, the extent to which they might crossreact with other organisms is not clear. We have been encouraged by the fact that a monoclonal antibody against mammalian hsp70 crossreacts with both major members of the hsp70 family in *Mytilus* (Figure 4). Most promising, we found that by Western blotting with this antibody we could detect the highly inducible hsp72 in mantle tissue exposed to heat shock, Cu, and TBT but not in the control.

Although perhaps not as higlhy inducible as hsp70, another good candidate for a tier I biomarker is the mitochondrial hsp60. Our own experiments have examined the accumulation of this stress protein because of the availability of an antibody probe with excellent crossreactivity for aquatic organisms. This hsp60 antibody was made against hsp60 from a moth[91] and has been shown to crossreact with hsp60 from a particularly wide variety of species ranging from the groEL gene product in *E. coli* to the hsp60 in rotifers,[12] *Mytilus* (Figure 5) and fathead minnow.[33]

Tier II: Stress Proteins as Stressor Specific Indices

In addition to providing information on an organism's integrated stress load, the relative synthesis of some stress proteins appears to be specific for different classes of stressors. These differences are particularly apparent in metabolic

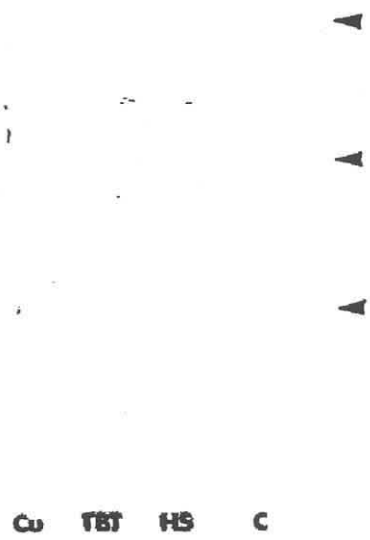

Figure 4. Western blot of *M. edulis* mantle tissue against a monoclonal antibody raised against mammalian hsp70 (from W. Welch). Tissues were exposed for 8 hr in vitro to copper, tributyltin, heat shock for 1 hr at 31°C and a control sample from left to right, respectively. Samples of equal protein were run on a 12% SDS gel before blotting. Arrows at the right designate prestained markers of approximately 194, 111, and 60 kDa, respectively. Cu = copper exposed sample, TBT = tributyltin exposed sample, HS = heat shock at 31°C, C = control.

labeling studies under acute conditions. The synthesis of hsp70 relative to other proteins, for example, is much greater in response to heat shock than in response to chemical inducers (Figures 2, 3). Also the inhibition of synthesis of other cellular proteins is less dramatic with chemical inducers than with heat shock. Although it is unclear if these differences will be of any practical use in environmental monitoring, it will at least allow us to determine if heat is a major stressor in specific acute situations or if organisms were accidently heat shocked during sample collection.

More promising as biomarkers for identifying exposure is the observation that certain contaminants elicit the synthesis of unique proteins.[92] The two most studied stressor specific stress proteins, heme oxygenase and metallothionein, have been discussed previously. In this section we will consider the potential of using unique translational patterns which correlate to different classes of stressors as biomarkers. At least initially, this approach would need to involve broad scale screening to examine changes in gene expression in response to different classes of environmental stressors. Cell lines from a variety of organisms would be exposed to individual contaminants and examined for increase synthesis of transcriptional or translational products. Once unique gene products were identified

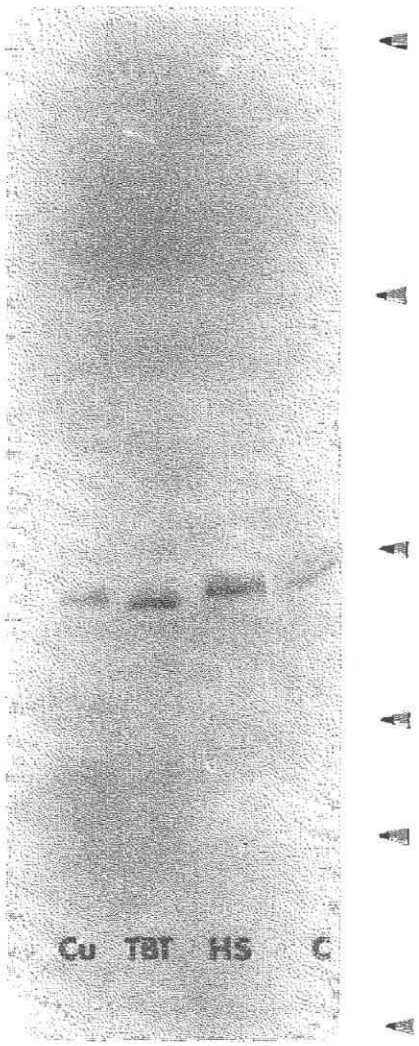

Figure 5. Western blot of *M. edulis* mantle tissue against a polyclonal antibody raised against hsp60 in moth.[91] Tissues and electrophoresis are described in Figure 4. Arrows represent prestained markers of approximately 194, 111, 60, 35, 26, 20, and 16 kDa, respectively. Cu = copper exposed sample, TBT = tributyltin exposed sample, HS = heat shock at 31°C, C = control.

in response to a class of stressor, an antibody could be raised against the unique protein for detecting in situ exposure.

This approach has been used extensively in facultative microorganisms where a set of genes and gene products that respond to a particular stress is referred to as a stimulon.[93] Although there is often overlap between stress proteins induced by such stressors as heat shock, anaerobiosis, oxidative stress, and starvation, unique proteins can also be attributed to each stressor.[14]

A few studies in eucaryotes have taken this approach to date and have generated promising results. *Neurospora* cells elicit different translational patterns when exposed to heat shock, arsenite, and oxidative stress.[13] A pattern of 11 proteins

is translated in response to heat shock, whereas in response to arsenite only the hsp70, hsp80, and a unique arsenite specific protein of approximately 40 kDa are translated. Hydrogen peroxide exposure leads to the synthesis of hsp70 and an oxidative stress-responsive protein (osp80).

Differential translation patterns are also found in both human and murine melanoma cells where hypothermia induces the synthesis of four stress proteins of 100, 90, and a doublet at 70 kDa.[11] In contrast, the heavy metals Cu and Cd, and thiolreactive agents induce the synthesis of these four proteins plus the 32 kDa stress protein, heme oxygenase. Lead induces an entirely different stress response in rat fibroblasts and epithelial cells.[40] Lead glutamate results in the increased synthesis of three stress proteins, grp100, grp80, and heme oxygenase, and does not induce hsp70 or metallothionein. In barley seedlings, salinity stress induces the synthesis of unique sets of low molecular weight stress proteins from the hsp20-30 family in a tissue-specific manner.[15] Other stress proteins, including hsp70, are not induced. Exposure of salivary gland cells to low-frequency electromagnetic fields alters translational patterns in a unique manner.[94] In addition to the five stress proteins which are also induced by heat shock, different subsets of stress proteins are induced in a frequency-dependent manner.

From the data currently available it appears that a limited number of specific genes may be activiated in a stressor specific manner resulting in unique patterns of translational products. Massive screening will of course be needed before we can accurately evaluate the potential of these unique patterns as biomarkers for exposure.

CONCLUSIONS

Several points need to be emphasized regarding the use of stress proteins as biomarkers. These proteins represent changes in gene expression in response to environmental variables which are part of the cellular strategy to protect itself from potential damage. Further, since cellular responses that would confer protection would be highly selected for in evolution, it is not surprising that stress responses are so highly conserved in procaryotes and eucaryotes alike. From a mechanistic viewpoint the strategy for selecting potential biomarkers which are based on the molecular mechanisms underlying protection from stress is a sound one with high potential for identifying environmentally relevant biomarker assays.

Much is known about regulation of gene expression of these stress responses and we are beginning to understand their role in the physiology of normal and stressed cells. However, very little is known about the environmental relevance of these responses in whole organisms exposed to stressors in their enviroment. Although preliminary data suggest that the accumulation of stress proteins can provide useful information for environmental monitoring and toxicological screening, much more research will be required before their usefulness can be

accurately evaluated. The response needs to be studied in a broad range of environmental contaminants at environmentally realistic concentrations. Research on the stress response is particularly needed in fish, aquatic invertebrates, and plants. Also the relationships between tissue level stress responses and impairment of function at the organismic level will be particularly important for this evaluation. The persistence of elevated stress protein concentrations in tissues of organisms exposed to contaminants in their environment needs to be fully explored under a wide range of environmental conditions. Finally, field studies must be undertaken to examine relationships between stress protein accumulation in organisms, other organismal and population stress indices, and chemical data on contaminant exposure.

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